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(21) International Application Number: PCT/DK97/00317 (22) International Filing Date: 23 July 1997 (23.07.97) (30) Priority Data: 8/208422 7 August 1996 (07.08.96) JP (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): MIYOTA, Yoshiaki [JP/JP]; Showa Denko K.K., Central Research Laboratory, 1-1, Ohnodai 1-chome, Midori-ku, Chiba-shi, Chiba 267 (JP). FUKUYAMA, Shiro [JP/JP]; Showa Denko K.K., Central Research Laboratory, 1-1, Ohnodai 1-chome, Midori-ku, Chiba-shi, Chiba 267 (JP). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: DOUBLE-STRANDED DNA WITH COHESIVE END(S), AND METHOD OF SHUFFLING DNA USING THE SAME (57) Abstract <p>To provide a method of mutation of DNAs, which is substantially different from the conventional methods applicable to naturally-existing DNAs, and also to provide useful genetic products to be produced by the use of thus-mutated DNAs. A DNA with a cohesive end comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, and (b) a single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, wherein the single-stranded DNA is linked to either one end of the double-stranded DNA to form a cohesive end; a method for producing it; a method of shuffling a DNA using it; a DNA and a DNA pool to be obtained by the shuffling method; a method for producing the DNA pool; and a genetic product to be obtained by expressing the genetic information existing in the DNA pool.</p>			

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Title: Double-stranded DNA with cohesive end(s), and method of shuffling DNA using the same

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INDUSTRIAL FIELD

The present invention relates to a double-stranded DNA with a cohesive end or cohesive ends having a desired sequence and a method for producing it, and also a method for shuffling
10 a DNA using DNA blocks with a cohesive end or cohesive ends, the DNA as shuffled according to the method, a DNA pool to be obtained according to the shuffling method, and also a genetic product to be produced by the use of the DNA pool.

15 BACKGROUND ART

One approach to protein engineering for improving naturally-existing proteins to modified ones which are more useful to human beings is to improve proteins through site-specific mutation, which has produced some results (Japanese Patent
20 Application Laid-Open No. 5-91876). However, this requires the clarification or identification of the stereostructure of the targeted protein, and much labor is needed for the analysis of the stereostructure. In addition, even though the stereostructure could be clarified or identified, there are still many
25 unknown matters for the relationship between the structure and the function with proteins. Therefore, it is still difficult to surely impart an intended function to the targeted protein.

In order to overcome these difficulties, a process comprising random mutation and screening and also evolutionary molecular engineering that utilizes the evolution of organisms have been being highlighted and said to be extremely useful
5 (Proc. Natl. Acad. Sci., USA, 83, 576 (1986)). However, the current methods are directed to the substitution of at most several amino acids.

In WO95/22625, disclosed is a method for forming novel genes by dividing a plurality of genes at random and homologously recombining them to reconstruct novel genes. However,
10 this is one method for forming chimera genes. The genes to be formed by this method are similar to the original genes, and the former shall have the essential base sequences of the latter.

15 Using such known methods, it is difficult to desire the impartation of some additional functions to organisms which they could not gain during the steps of their evolution. In order to obtain genetic products, of which the functions are greatly different from those of naturally-existing substances
20 such as proteins, it is believed effective to prepare a pool of nucleic acids having significantly different base sequence spaces from those existing naturally, and to produce from them genetic products having the intended functions.

One method for this may be to prepare a nucleic acid pool
25 that covers all base combinations. However, even the total number of the base sequences that may code for a relatively small protein with 100 amino acids (300 bp) is an enormous

number of 4^{300} (about 10^{180}), and it is in fact impossible to prepare the nucleic acid pool that may cover all of them.

For proteins of some kinds, their sub-structures which are referred to as modules were specifically noted, and an attempt
5 was made to change the sequencing of the base sequence blocks corresponding to the individual modules to thereby produce mutants having different module sequences (Viva Origino, Vol. 23, No. 1 (1995) 86-87). In this attempt, however, the base sequences were re-sequenced merely individually for the
10 individual mutants. No one has heretofore attempted the formation of a nucleic acid pool covering all re-sequenced molecules and the collection of genes capable of expressing products having intended properties from the pool.

Utilizing restriction enzymes, it is possible to prepare a
15 nucleic acid pool including various molecules by blending several kinds of DNA blocks having the same cohesive end or blunt end followed by ligating them at random, and to select from this pool some molecules having desired properties. According to this method, however, the DNAs to be used must have prede-
20 termined restriction enzyme recognizing sites. Even though the DNAs have such restriction enzyme recognizing sites, there is an extremely small probability that the sites are positioned at the desired sites. In this method, in addition, the both ends of the blocks must be of the same type, and there is a high
25 probability that the blocks are therefore self-ligated. A means of forming the restriction enzyme recognizing sites through site-specific mutation may be taken in order to overcome these problems. However, the matter as to whether or not

the blocks can be ligated in accordance with the predetermined frame is likely much governed by chance. That is, the matter as to whether or not a desired protein can be produced without misreading the reading frame of the codon shall be governed by
5 chance. Therefore, the method is extremely inefficient.

The subject matter of the present invention is to provide a method for efficiently obtaining base sequences that exist in spaces greatly different from those of naturally-existing base sequences, and also to provide genetic products to be obtained
10 by expressing, as genes, the nucleic acid sequences that are obtained in that manner and that do not exist naturally.

The sequence space of a gene includes the full-length sequence thereof to be theoretically constituted by a combination of four bases, A, G, C and T. For example, a base sequence that codes for a protein composed of a number "n" of amino
15 acids shall be constructed by selecting and sequencing any desired one of the four bases for a total of $3n$ -times, therefore including 4^{3n} combinations. Accordingly, a protein composed of 100 amino acids shall include different base sequences of about
20 10^{18} types as so mentioned hereinabove.

In fact, there is no limitation for the number of amino acids that constitute proteins. Therefore, the sequencing spaces for proteins shall extend unlimitedly. During the steps of evolution of organisms, only a part of such sequencing spaces
25 have been examined, and there is a great probability that some sequences coding for proteins which may have some extremely excellent functions could exist in the other great sequencing spaces. The protein engineering studies which have been and

are being made in many laboratories and institutes at present are essentially directed to the creation of novel proteins having functions superior to those of naturally-existing proteins, and one essential approach made therein to this purpose is to substitute amino acids in existing sequences, as so mentioned hereinabove. However, the amino acid substitution is nothing but the essential means that organisms have carried out during the steps of their evolution or, that is, such is the imitation of organisms and is to search only around the sequences that organisms already examined. In addition, there is a probability that the sequences thus obtained will be those that were already weeded out in the past.

We, the present inventors have considered that, in order to be greatly apart from the sequencing spaces that organisms already examined, if we carry out such matters that could not have been carried out by organisms, the purpose will be attained. We know that the division of a gene into several blocks followed by the change in the sequencing of the thus-divided blocks, if occurred in organisms, shall kill the organisms. Therefore, we have concluded that this method is suitable for our purpose. Having thus concluded, we, the present inventors have assiduously studied various matters relating to this method and, as a result, have found a method of forming a desired cohesive end or ends on a desired DNA. Utilizing this method, we have succeeded in a method of dividing a gene into several blocks and re-sequencing them into different sequences and also in a method of producing a molecule pool including such different base sequences existing in different

sequencing spaces, and thus have completed the present invention.

Accordingly, the present invention provides the following:

1) A DNA with a cohesive end comprising (a) a double-
5 stranded DNA having the same sequence as that of a part of a
gene, and (b) a single-stranded DNA having a base sequence that
exists on said gene at the site not adjoining the part cor-
responding to said double-stranded DNA or a base sequence which
said gene does not have, wherein the single-stranded DNA is
10 linked to either one end of the double-stranded DNA to form a
cohesive end.

2) A DNA with cohesive ends comprising (a) a double-
stranded DNA having the same sequence as that of a part of a
gene, (b) a first, single-stranded DNA having a base sequence
15 that exists on said gene at the site not adjoining the part
corresponding to said double-stranded DNA or a base sequence
which said gene does not have, and (c) a second, single-
stranded DNA having a base sequence that exists on said gene at
the site adjoining the part corresponding to said double-
20 stranded DNA, wherein the second, single-stranded DNA is linked
to said double-stranded DNA at one end corresponding to said
adjoining site, while the first, single-stranded DNA is linked
thereto at the other end of the complementary strand opposite
to said end, thereby forming cohesive ends.

25 3) The DNA with a cohesive end or cohesive ends according
to the previous 1) or 2), wherein the single-stranded DNA has a
length of 2 bases or more.

4) The DNA with a cohesive end or cohesive ends according to any one of the previous 1) to 3), wherein the cohesive end/ends is/are positioned at the 3'-terminal/terminals.

5) A method for producing a DNA with a cohesive end or
5 cohesive ends, wherein a part of a DNA, as a template, and an oligonucleotide containing at least one ribonucleotide, as a primer, are subjected to DNA polymerase reaction to prepare a double-stranded DNA, then the ribonucleotide(s) is/are removed through enzymatic reaction or chemical reaction, and the nu-
10 cleotide(s) remaining at the 5'-terminal(s) of the site(s) at which said ribonucleotide(s) existed are removed.

6) A method for producing the DNA with a cohesive end of the previous 1), comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the
15 same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide
20 (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a tem-
25 plate, and the linked oligonucleotide as obtained in the previous step a), as a primer;

c) a step of removing the ribonucleotide from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotide remaining at the 5'-terminal of the site at which said ribonucleotide existed.

7) A method for producing the DNA with cohesive ends of the previous 2), comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and (i) the linked oligonucleotide as obtained in the previous step a) and (ii) an oligonucleotide which is a complementary strand of an oligonucleotide existing on the gene at the site separated from said oligonucleotide-corresponding part by at least 3 bases or more toward the 3'-terminal and which contains at least one ribonucleotide, as primers;

c) a step of removing the ribonucleotides from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotides remaining at the 5'-terminals of the sites at which said ribonucleotides existed.

8) A method for shuffling a DNA, comprising dividing a DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

9) A method for shuffling a DNA, comprising applying the method of any one of the previous 5) to 7) to various sites of a DNA, thereby dividing the DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, at least one block of which shall have a cohesive end that is complementary to the cohesive end of another block not having been directly adjacent to said one block on the original DNA, followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

10) The shuffling method according to the previous 8) or 9), wherein the DNA is divided into 3 or more blocks.

11) The shuffling method according to any one of the previous 8) to 10), wherein the blocks are ligated together using a DNA ligase.

12) A DNA as shuffled according to the method of any one of the previous 8) to 11).

[0016]

13) The DNA according to the previous 12), wherein a gene coding for an enzymatic function or a control gene for the gene is shuffled.

14) The DNA according to the previous 13), wherein the gene is a gene that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

5 15) The DNA according to the previous 13) or 14), wherein the gene is one derived from prokaryotes.

16) The DNA according to the previous 15), wherein the gene is one derived from bacillus bacteria.

17) The DNA according to the previous 16), wherein the
10 gene is a protease API21 gene.

18) A DNA pool containing plural kinds of DNAs having different structures that are obtained according to the shuffling method of any one of the previous 8 to 11).

19) The DNA pool according to the previous 18), which
15 contains 10 or more kinds of DNAs.

20) A method for producing a DNA pool, comprising applying the method of any one of the previous 5) to 7) to various sites of a template DNA to thereby prepare a mixture of DNA blocks each having a cohesive end or cohesive ends that
20 satisfies the following conditions, followed by ligating these into any desired sequences:

Condition 1: Each block has a double-stranded site having the same sequence as that of a part of the template DNA.

Condition 2: At least two of the blocks that constitute
25 the block mixture further have, in addition to said double-stranded site, a single-stranded site (cohesive end) that is complementary to the cohesive end of blocks that are not directly adjacent to said blocks on the template DNA.

Condition 3: The block mixture contains at least two different blocks which are the same in the double-stranded site but are different only in the single-stranded site and which satisfy the condition 2.

5 21) The method for producing a DNA pool according to the previous 20), wherein the template DNA is a gene that codes for an enzymatic function or a control gene DNA for the gene.

22) The method for producing a DNA pool according to the previous 21), wherein the template DNA is a gene DNA that codes
10 for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

23) The method for producing a DNA pool according to the previous 22), wherein the template DNA is one derived from
15 prokaryotes.

24) The method for producing a DNA pool according to the previous 23), wherein the template DNA is one derived from bacillus bacteria.

25) The method for producing a DNA pool according to the
20 previous 24), wherein the template DNA is a protease API21 gene.

26) The method for producing a DNA pool according to any one of the previous 20) to 25), wherein the DNA blocks are ligated together using a DNA ligase.

25 27) A genetic product to be obtained by expressing the genetic information on DNA molecules that exist in the DNA pool of any one of the previous 18) to 26).

Now the present invention is described in detail hereinunder.

DNA with Cohesive End(s)

5 The present invention provides a DNA with any desired cohesive end or ends (herein referred to as "DNA with cohesive end(s)" unless otherwise specifically indicated). The cohesive end as referred to herein indicates a single-stranded site as protruded from the end of a double-stranded DNA. Such a
10 cohesive end may be formed when a DNA is cleaved with a restriction enzyme such as EcoRI. In this case, however, the base sequence of the thus-formed cohesive end is defined, depending on the restriction enzyme used, and its length is generally composed of several bases or so. If a naturally-
15 existing DNA is cleaved with a restriction enzyme, the sequence of the resulting double-stranded part of the DNA is also limited to the region as sandwiched between the restriction enzyme recognizing sites. As opposed to this, the DNA with cohesive end(s) of the present invention may have a structure in
20 which a cohesive end or cohesive ends having a desired length and a desired sequence is/are added to the end or ends of a double-stranded DNA having a desired sequence.

As has been mentioned hereinabove, the sequence of the double-stranded part of the DNA with cohesive end(s) of the
25 present invention is not specifically defined. For example, the sequence may be the same as that of a part of a gene. Though not also specifically defined, its length may be generally composed of 50 base pairs (bp) or more, preferably 45 bp

or more. The sequence of the cohesive end is not also specifically defined, but in order to prevent the self-ligation thereof in various reactions, it is preferable that the sequence does not form a stem structure. The "sequence to form
5 a stem structure" as referred to herein includes, for example, AATT, which shall have just the same sequence as that of its complementary strand (TTAA). The length of the cohesive end may be generally 2 bp or more, preferably from 15 bp to 30 bp. If the cohesive end is too long, it may form a secondary struc-
10 ture whereby the intermolecular annealing will be difficult. However, if it is too short, its melting temperature (T_m) is low and the annealing will be unstable.

The cohesive end may be linked to either the 3'-terminal or the 5'-terminal of the double-stranded DNA, but is preferably linked to the 3'-terminal thereof. The cohesive end may
15 be linked to either only one terminal of the double-stranded DNA or the both terminals thereof.

Method for Producing DNA with Cohesive End(s)

20 The DNA with cohesive end(s) of the present invention can be produced typically according to a method comprising the following steps a) to d). The method mentioned below is directed to the production of a DNA with a cohesive end, which has a structure to be represented by a formula (2):

wherein \times and c are desired sequences; a and a are sequences that are complementary to \times and c, respectively; and a is a sequence of a cohesive end, and which is based on a double-stranded DNA (template DNA) having a structure to be represented by a formula (1):

(1)

wherein \times , c, a, a , and a have the same meanings as above.

10 However, the present invention is not limited to only the production illustrated herein, but other DNAs with cohesive end(s) having other structures can also be produced in the same manner as below according to the present invention.

15 Step a)

(a-1) Preparation of Oligonucleotide:

First, the part that shall be selected as the double-stranded part of the intended DNA with a cohesive end is defined on a template DNA. An oligonucleotide, a, which is complementary to its terminal, \times , and an oligonucleotide, c, having the same sequence as that of the other terminal, c, are prepared. \times and c each may have a sequence having a base length of from 15 to 30 bp or so.

On the other hand, prepared is an oligonucleotide, b, which is complementary to the sequence to be prepared by removing one base (this is referred to as X) from the 5'-terminal of the sequence of the intended cohesive end, a .

The base sequence, \mathcal{W} , may be a part of the above-mentioned DNA or may be any other sequence that the DNA does not have.

These oligonucleotides, a, b and c, may be prepared by any methods. If their sequences are previously known, they can be synthesized, using a known DNA synthesizer.

(a-2) Preparation of Ribonucleotide-Containing Fragments:

Next, the oligonucleotides, a and b, are linked together via a ribonucleotide. This linkage can be attained by ordinary synthesizing methods. For example, it can be attained according to the process mentioned below.

First, a phosphoryl group is added to the 5'-terminal of the oligonucleotide, a, according to the reaction of the following formula (3):

15

(3)

wherein (P) is a phosphoryl group.

This reaction can be effected by the action of a polynucleotide kinase. ATP is used in an amount of from 2 to 10 times or so, by mol, relative to the oligonucleotide, a. The reaction temperature may be from 30 to 40°C or so. The reaction time may be from 10 minutes to 1 hour or so. Most suitably, the pH is from 7 to 9 or so. After the addition of the phosphoryl group thereto, the oligonucleotide is represented by a'.

[0024]

On the other hand, a ribonucleotide is added to the 3'-terminal of the oligonucleotide, b, according to the reaction of the following formula (4):

(4)

wherein X is any one of ATP, GTP, CTP and UTP; (rX) is a ribonucleotide.

This reaction can be effected by the action of, for example, a terminal deoxynucleotidyl transferase. For the nucleoside triphosphate (XTP) to be used herein, is selected a ribonucleotide that corresponds to the base X in the previous step (a-1). The nucleoside triphosphate is used in an amount of from 2 to 10 times, by mol, relative to the oligonucleotide, b. The reaction temperature may be from 30 to 40°C or so. The reaction time may be from 30 minutes to 2 hours or so. After the addition thereto, the oligonucleotide is represented by b'. The sequence of b' is complementary to the sequence, \bar{a} .

The thus-obtained oligonucleotides, a' and b', are mixed, whereby the 5'-terminal (phosphoryl group) of a' is bonded to the 3'-terminal (hydroxyl group) of the ribonucleotide of b', according to the reaction of the following formula (5):

(5).

This reaction can be effected by the action of an RNA ligase in the presence of ATP and divalent metal ions (Japanese Patent Application Laid-Open No. 5-292967). Divalent metal ions useful in this reaction include, for example, magnesium ions and manganese ions, but preferred are magnesium ions. As the ligase, employable is an RNA ligase. The RNA ligase is an enzyme to catalyze the ligation of the hydroxyl group at the 3'-terminal and the phosphoryl group at the 5'-terminal, and this also efficiently catalyzes the ligation of a

polydeoxyribonucleotide having a ribonucleotide only at its 3'-terminal and a polydeoxyribonucleotide with a 5'-terminal phosphoryl group. Preferably used is a T4 RNA ligase. The reaction is generally effected in a buffer, at a pH of from 7 to 9 and at a temperature of from 10 to 40°C, over a period of from 30 to 180 minutes. For example, the oligonucleotides may be reacted in a solution comprising 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 0.1 mM ATP, 10 mg/liter BSA, 1 mM hexaammine cobalt chloride (HCC) and 25 % polyethylene glycol 6000, at 25°C for 60 minutes or longer.

Step b)

Using the DNA containing the sequence, \times , as prepared in the previous step (a-1), as a template, and using the linked oligonucleotide, b'-a', as prepared in the previous step (a-2), as a primer, prepared is a double-stranded DNA through DNA polymerase reaction. In general, a double-stranded DNA containing the sequence, \times , and a sequence, Φ , on their strands is subjected to thermal or alkaline denaturation to give single-stranded DNAs, which are added to the primer of b'-a' and subjected to PCR with the oligonucleotide, c, as prepared in the previous step (a-1). The primer annealing condition and the polymerase reaction condition to be employed herein may be the same as those in ordinary polymerase reaction. The DNA polymerase to be employed herein may be any and every enzyme that can catalyze the DNA chain-extending reaction, which includes, for example, Taq polymerase, Klenow fragment, DNA polymerase I, etc. As a result of this reaction, obtained is a

double-stranded DNA with blunt ends, which is represented by the a formula (6):

(6).

Step c)

5 Next, the ribonucleotide is removed from the double-stranded DNA through enzymatic reaction or chemical reaction. One example of useful enzymes is a ribonuclease. The reaction is generally effected at a pH of from 6 to 8 or so, at from 30 to 70°C or so, over a period of from 10 to 60 minutes or so.
10 As non-enzymatic chemicals usable herein, mentioned are sodium hydroxide and the like. As a result of this reaction, obtained is a partly-discontinuous, double-stranded DNA of the following formula (7), in which the part corresponding to the above-mentioned base, X, has been deleted.

15

(7).

Step d)

After the above step, the nucleotide existing at the 5'-terminal of the above-mentioned deletion is removed. To remove
20 this nucleotide, for example, the double-stranded DNA, from which the ribonucleotide has been removed in the previous step c), is heated at from 50 to 90°C or so. The polynucleotide that has been separated from the strand through this reaction can be removed, using, for example, a span column or the like.
25 Thus is obtained the double-stranded DNA with a cohesive end of the above-mentioned formula (2).

In the process mentioned above, obtained is a double-stranded DNA with a cohesive end only at its one 3'-terminal.

In the same manner as this, also obtainable is a double-stranded DNA with cohesive ends at the both 3'-terminals.

In the above-mentioned process, a desired sequence, ω , which does not adjoin the sequence, χ , in the template DNA was introduced into the DNA to form the cohesive end. Apart from this, it is also possible to introduce thereinto an additional oligonucleotide that adjoin the sequence in the template DNA to form another cohesive end. For example, in the embodiment mentioned above, an oligonucleotide, c' , which is different from the oligonucleotide, c , in that its 3'-terminal deoxyribonucleotide is substituted with a ribonucleotide, may be used as the primer in place of the oligonucleotide, c , to prepare a double-stranded DNA with two cohesive ends of a formula (8):

(8).

Method of Shuffling DNA

The present invention also provides a method of shuffling a DNA, which is characterized by using DNAs with cohesive end(s). The terminology "shuffling" as referred to herein indicates the operation of dividing a DNA into plural blocks followed by re-sequencing them into a desired, different sequence.

For example, where one DNA has a sequence composed of a number, n , of blocks, as represented by a formula (9):

$A - a_1 - a_2 - \dots - a_n - B$ (9)

wherein the starting end A and/or the terminal end B may be omitted,

this may be shuffled according to the present invention to give a different DNA to be represented by a formula (10):

$$A - a_1' - a_2' - \dots - a_x - B \quad (10)$$

wherein a_1' , a_2' , \dots , a_x are blocks that are
 5 independently selected from the group of a_1 , a_2 , \dots , a_n ; and the total number of the blocks a_1' , a_2' , \dots , a_x may not be the same as the total number of the blocks a_1 , a_2 , \dots , a_n .

The principle of the DNA shuffling of the present
 10 invention which utilizes DNAs with cohesive end(s) is graphically illustrated in Fig. 1. In Fig. 1, the DNA is shuffled at the intermediate part, $p_1 - p_2 - p_3$ (the uppermost row) into $p_3 - p_1 - p_2$ (the lowermost row), without changing the both ends p_A and p_B . This shuffling operation is useful as a method
 15 for obtaining gene sequences that have not heretofore existed naturally, without changing the sequences of the promoter and the terminator.

Concretely, the above-mentioned method of preparing DNAs with cohesive end(s) is applied first to the parts p_A , p_1 , p_2 ,
 20 p_3 and p_B constituting the template DNA, to thereby prepare DNA blocks, a_1 , a_2 and a_3 , each having the structure with two cohesive ends (formula (8)), and DNA blocks, A and B , each having the structure with one cohesive end (formula (2)). The cohesive ends, a_A , a_{1f} , a_{2f} and a_{3f} , are formed by removing the
 25 corresponding complementary strand from the blocks, p_A , p_1 , p_2 and p_3 , respectively.

The cohesive ends, a_{1r} , a_{2r} , a_{3r} and a_B , are designed according to the desired sequence to be prepared after the shuffling. In the embodiment of Fig. 1, the end, a_{1r} is designed to be a complementary strand to the end, a_{3f} , and the 5 block, a_1 is ligated to the block a_3 after the shuffling. The ligation is conducted, using a DNA ligase in the presence of ATP. The type of the DNA ligase to be employed herein is not specifically defined. In this embodiment, since the single-stranded part of each cohesive end is long, it is unnecessary 10 to employ the ordinary reaction at 16°C, but a thermophilic DNA ligase is advantageously employed.

In the embodiment of Fig. 1, a_{2r} , a_{3r} and a_B are designed to be the complementary strands to a_{1f} , a_A and a_{2f} , respectively, in the same manner as above. As a result of the shuffling, a sequence having a structure of A - a_3 - a_1 - a_2 - B is 15 finally obtained. This is seemingly the same as the re-sequenced order of p_A - p_3 - p_1 - p_2 - p_B to be obtained by dividing the original DNA into the constitutive blocks p_1 , p_2 , p_3 , p_A and p_B , followed by re-sequencing these into a different 20 sequence.

Any other desired sequences can be realized in the same manner as above. If the block, A or B, is made to have two cohesive ends, while the other blocks are made to have one cohesive end, it is possible to obtain still different sequences 25 through shuffling where the latter blocks with one cohesive end are positioned at the terminals.

In the shuffling of the invention, it is also possible to introduce foreign DNA block(s) with cohesive end(s), which are

not in the original gene, into the gene DNA. For example, it is possible to shuffle two or more gene DNAs. In this case, the terminal of one gene, for example, the block A in the above-mentioned embodiment, may be processed into a DNA block 5 with two cohesive ends, if desired.

The blocks, which are the units to be shuffled, are oligonucleotides or polynucleotides composed of 2 or more nucleotides (hereinafter referred to as "oligonucleotides"). In general, these are preferably composed of 30 or more 10 nucleotide units, more preferably 45 or more nucleotide units. The uppermost limit of the block length is not specifically defined, provided that the block length is shorter than the length of one gene. If, however, the block length is too large, the re-sequenced DNA to be obtained by the shuffling 15 shall have many non-mutated base sequence parts. Therefore, in general, the block length is preferably within the range of from 10 to 35 % of the length of a gene.

Where the gene to be shuffled is a gene that codes for a protein, it is desirable that the gene blocks, oligonucleotides 20 have the same reading frame before and after the division. Namely, the gene blocks to be shuffled are desirably so designed that they are translated to always give the corresponding amino acid sequences, irrespective of their relative positions in the shuffled sequence. For this, the double-stranded parts 25 and the cohesive ends shall be selected for their codon units in accordance with the reading frame of the gene DNA to be shuffled.

Needless-to-say, it is unnecessary to conduct the division into segment blocks with genetic meanings. Namely, it is unnecessary to conduct the division of the gene DNA into the constitutive exons or segment blocks that correspond to the 5 domains or modules of the protein which the gene DNA codes for. There is a probability that the shuffling at such sites would have been examined in the natural world in the past. In order to obtain base sequences that have not heretofore been examined in the natural world, it is desirable that the division of the 10 gene DNA is effected inside the constitutive exons or at the sites corresponding to the inside of the domains or modules of the protein which the gene DNA codes for.

Employing such means, therefore, it is possible to obtain proteins which have different structures as a whole from those 15 of natural proteins but which partly contain amino acid sequences that have been confirmed to be useful in the natural world. Accordingly, the probability of obtaining useful proteins by such means is enlarged, as compared with the means of synthesizing proteins totally at random.

20 The kind of the gene to be shuffled according to the present invention is not specifically defined. Employable herein is any and every gene that is composed of polynucleotide chains and contains a coding region necessary for expressing a protein or RNA. The nucleotide unit may contain any molecule of deoxy- 25 ribonucleotides or ribonucleotides. For the purpose of finding out useful base sequences, preferred are genes coding for proteins, especially enzymes, or control genes for enzymatic functions. Examples of such enzymes include proteases, lipa-

ses, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

The kind of the gene to which the present invention is directed is not specifically defined but shall be such that, when it is introduced into a suitable host, the host can produce the genetic product through expression of the gene. As examples, referred to are genes as cloned from living organisms, artificially synthesized genes, and even genes as cloned from living organisms and artificially mutated. For the genes derived from living organisms, employable are prokaryotes with definite enzyme producibility. As examples of such prokaryotes, mentioned are bacillus bacteria. One example of the genes derived from such bacteria is a protease API21 gene derived from *Bacillus* NKS-21 (FERM BP-93-1) (Japanese Patent Application Laid-Open No. 5-91876, Sequence Number 1).

DNA Pool

The present invention also provides a DNA pool to be obtained according to the above-mentioned shuffling method. The "DNA pool" as referred to herein means a high-density mixture of two or more DNAs. The DNA pool of the present invention can contain a particular number or more, for example, 10 or more different DNA molecules having different structures. It is desirable that, when the mixture, DNA pool is directly used in biochemical operation or reaction, it is in such a form that all the plural nucleic acid components constituting it can be reacted. However, the form of the mixture, DNA pool is not

specifically defined, and the DNA pool may be either in solution or dry mixture.

To produce the DNA pool, for example, a plurality of cohesive ends for each block are prepared in the above-mentioned shuffling process. Referring to the embodiment of Fig. 1, for example, when, for the cohesive end a_{1r} of the block a_1 , complementary strands to the other cohesive ends, a_A and a_{2f} , are prepared in addition to the complementary strand to a_{3f} , then DNAs of $A - a_1 - a_2 - B$ and $A - a_1 - a_2 - a_1$ can be obtained. If a complementary strand to the other cohesive end a_{1f} of a_1 is added, it is also possible to produce other DNAs comprising a series of the same blocks, such as $A - a_1 - a_1 - a_1$.

In the same manner, for the cohesive ends of a_2 and a_3 , if oligonucleotides that are complementary to the cohesive ends of the other blocks or complementary to the other cohesive end of themselves are added, other sequences comprising these can be produced.

In general, a DNA is divided into blocks of $a_1, a_2, a_3, \dots, a_n$. Then, each block is processed to have a cohesive end or cohesive ends according to the above-mentioned process. The cohesive ends are designed to be oligonucleotides that are complementary to the cohesive ends of the other blocks or are complementary to the other cohesive end of themselves. All or a part of the thus-obtained DNA blocks are mixed and ligated to each other, thereby producing a nucleic acid pool containing different nucleic acids composed of the blocks as differently sequenced at random.

Expression of Genetic Information in Shuffled DNA or DNA Pool

The thus-shuffled, single or mixed, double-stranded DNAs are blunted. The blunting may be omitted, if DNA blocks with one cohesive end are positioned at the ends of the shuffled, 5 double-stranded DNA. For example, the 5'-terminal of the sequence containing a DNA block with a predetermined promoter sequence, which is based on the direction of the promoter, is not made to have a cohesive end but is made to have a blunt end, while the 3'-terminal of the sequence containing a DNA block 10 with a predetermined terminator sequence, which is based on the direction of the terminator, is not made to have a cohesive end but is also made to have a blunt end. In that manner, it is possible to directly obtain a gene in which the blocks of the intended gene have been shuffled between the promoter and the 15 terminator, without blunting it. After this, the thus-shuffled DNA is inserted into a desired vector, preferably an expression vector such as pKK223-3, using a DNA ligase. the promoter sequence and the terminator sequence to be in the shuffled DNA are not limited to only one each, but a plurality of promoter 20 sequences and terminator sequences may be therein.

If desired, the polynucleotide blocks positioned at the both ends of the shuffled DNA may be designed to have suitable restriction enzyme recognizing sites. In this case, the DNA may be ligated to a suitable vector, using the defined re- 25 striction enzymes.

Next, the vector library thus produced in the manner mentioned above is introduced into a suitable host, in which the genetic information is expressed. Thus, the intended gene-

tic product with favorable properties and also the gene coding for it can be obtained. Any and every ordinary host can be used herein. Preferred examples of the host include cells of *E. coli*, bacillus bacteria, yeasts, and lactic acid bacteria.

5 If desired, *in-vitro* transcription systems and translation systems are also employable herein. In those cases, the genetic information can be expressed even when the gene is not ligated to a vector.

The "genetic information" as referred to herein indicates
10 the information on a gene which is carried by a DNA and which is translated into a protein or is transcribed into RNA in a suitable living body by the DNA for itself or after having been ligated to any other DNA or RNA.

The genetic information that is expected to be expressed
15 according to the method of the present invention is not specifically defined, but includes, for example, those on various genetic products, such as enzymes, antibodies, hormones receptor proteins and ribozymes, and those on various control functions of, for example, operators, promoters and attenua-
20 tors.

Examples

Now, the present invention is described in detail hereinunder with reference to the following examples, which,
25 however, are not intended to restrict the scope of the present invention.

Example 1: Production of DNA Pool

A nucleic acid pool was produced in accordance with the process mentioned below, based on the wild-type alkali protease (Japanese Patent Application-Laid Open No. 5-91876) as cloned from a protease API21 (*Bacillus* NKS-21; FERM BP-93-1) having a sequence of Sequence Number 1.

(1) Step a): Preparation of Oligonucleotide Blocks for Primer
(1-1) Synthesis of Oligonucleotide Blocks:

Using an automatic DNA synthesizer, Model 392 (manufactured by Perkin Elmer Co.), synthesized were 14 oligonucleotides; oligo FW (Sequence Number 2), oligo RV (Sequence Number 3), oligo 1r (Sequence Number 4), oligo 1b (Sequence Number 5), oligo 1a (Sequence Number 6), oligo 2r (Sequence Number 7), oligo 2b (Sequence Number 8), oligo 2a (Sequence Number 9), oligo 3r (Sequence Number 10), oligo 3b (Sequence Number 11), oligo 3a (Sequence Number 12), oligo 4r (Sequence Number 13), oligo 4b (Sequence Number 14), oligo 4a (Sequence Number 15) and oligo A (Sequence Number 16). These are parts of the base sequence of API21 (Japanese Patent Application Laid-Open No. 5-91876) (including complementary strands), or oligonucleotides containing a part of the base sequence. However, the sequence of oligo 4a is to follow glutamine of Sequence Number 1 and, and this contains a termination codon of the gene. These oligonucleotides were so designed that they might be the best when the oligo A was overhung on the 3'-terminal of the amplified DNA in the experiment to follow hereinunder, using a Taq polymerase.

These oligonucleotides were synthesized in a DM trityl-on condition (that is, while the 5'-hydroxyl group was protected with dimethoxytrityl group), and purified through an OPC column. The reagents used herein were obtained from Perkin
5 Elmer Co.

(1-2) Addition of Ribonucleotide to Blocks:

Next, 500 pmols of oligo 1r, 1 nmol of ATP and 10 units of terminal deoxynucleotidyl transferase were added to a standard solution comprising:

10 50 mM Tris-HCl buffer (pH 8.0)

10 mM MgCl₂

5 mM DTT (dithiothreitol)

25 % PEG 6000

1 mM HCC (hexaammine cobalt chloride)

15 10 µg/ml BSA (bovine serum albumin),

to thereby make 10 µl in total. The resulting solution was left at 37°C for 1 hour.

Oligo 2r, oligo 3r, oligo 4r, oligo 1b, oligo 2b, oligo 3b and oligo 4b were processed in the same manner as above. These
20 four polynucleotides thus formed are referred to as oligo 1r', oligo 2r', oligo 3r', oligo 4r', oligo 1b', oligo 2b', oligo 3b' and oligo 4b'.

(1-3) Phosphorylation:

500 pmols of oligo 1a, 1 nmol of ATP and 10 units of
25 polynucleotide kinase were dissolved in the standard solution having the same composition as above to make 10 µl in total. The resulting solution was left at 37_C for 1 hour. Oligo 2a, oligo 3a and oligo 4a were processed in the same manner as

above. These polynucleotides thus formed are referred to as oligo 1a', oligo 2a', oligo 3a' and oligo 4a'.

(1-4) Ligation of Oligonucleotide Blocks:

500 pmols of oligo 1a', 100 pmols of oligo 1b', 100 pmols
5 of oligo 2b', 100 pmols of oligo 3b', 100 pmols of oligo 4b',
which had been obtained in the above, as well as 1 nmol of ATP
and 50 units of T4 RNA ligase were added to the same standard
solution as that mentioned above to make 10 μ l in total, and
these were reacted at 25°C for 4 hours.

10 The other combinations, oligo 2a' with oligo 1b', oligo
2b', oligo 3b' and oligo 4b'; oligo 3a' with oligo 1b', oligo
2b', oligo 3b' and oligo 4b'; and oligo 4a' with oligo 1b',
oligo 2b', oligo 3b' and oligo 4b', were also reacted in the
same manner as above. A mixture of the four polynucleotides
15 thus formed as a result of this reaction, oligo 1a' ligated to
oligo 1b', oligo 2b', oligo 3b' and oligo 4b', is referred to
as oligo 1M; a mixture of the four polynucleotides, oligo 2a'
ligated to oligo 1b', oligo 2b', oligo 3b' and oligo 4b', is
referred to as oligo 2M; a mixture of the four polynucleotides,
20 oligo 3a' ligated to oligo 1b', oligo 2b', oligo 3b' and oligo
4b', is referred to as oligo 3M; and a mixture of the four
polynucleotides, oligo 4a' ligated to oligo 1b', oligo 2b',
oligo 3b' and oligo 4b', is referred to as oligo 4M.

(2) Steps b) to d): Formation of Gene Blocks

25 A template, plasmid pSDT812 (Japanese Patent Application
Laid-Open No. 1-141596), which had been prepared by inserting,
into the ClaI cleaving site of pHSG396, the gene of the wild-
type alkali protease as cloned from *Bacillus* NKS-21, was

subjected to PCR with primers, oligo 1M and oligo 2r'. The gene fragment as amplified through this reaction was treated with a ribonuclease, and then heated at 80_C for 5 minutes, whereby the polynucleotide(s) positioned at the 5'-terminal of the ribonucleotide existing in the both strands or one strand was/were removed. As a result of this, prepared was a gene block with cohesive end(s). This gene block is referred to as block 1M.

The other four combinations, oligo 2M and oligo 3r', oligo 3M and oligo 4r', oligo 4M and oligo RV, and oligo FW and oligo 1r', were processed in the same manner as above. These blocks thus prepared are referred to as block 2M, block 3M, block B, and block F, respectively.

15 Example 2: Shuffling

Block 1M, block 2M, block 3M, block B and block F of the same amount were blended and ligated together, using Pfu DNA ligase.

After the ligation, the reaction mixture was subjected to agarose gel electrophoresis, through which was collected the DNA fragment of about 1.5 kbp.

Example 3: Identification of Nucleic Acid Pool

The thus-collected DNA of about 1.5 kbp was digested with restriction enzymes, EcoRI and BamHI, then mixed with a plasmid, pHY300PLK (manufactured by Yakult Honsha Co.), which had been digested with restriction enzymes, EcoRI and BamHI and processed with an alkali phosphatase, and thus ligated

together, using a ligation kit (manufactured by Takara Shuzo Co.). Using the resulting DNA, cells of *E. coli* JM105 were transformed, from which were selected tetracycline-resistant transformants. From these transformants, plasmid DNAs were
5 extracted, purified and analyzed according to ordinary methods. Thus were obtained 97 clones with a DNA of 1.5 kbp as inserted between the EcoRI and BamHI recognizing sites of pHY300PLK.

The base sequences of these DNAs thus obtained in the manner mentioned above were sequenced to analyze how block 1M,
10 block 2M, block 3M, block F and block B were ligated in what order or, that is, how these were shuffled. As in the principle, block F was positioned at the first site while block B at the fifth site, and block 1M, block 2M and block 3M were shuffled between the two. Table 1 shows different types of
15 shuffling, and the number of clones with each type.

Table 1

Type of Shuffling	Number of Clones	Type of Shuffling	Number of Clones
111	2	223	2
112	5	231	5
113	2	232	2
121	3	233	3
122	4	311	2
123	7	312	6
131	4	313	5
132	5	321	7
133	3	322	2
211	1	323	5
212	5	331	2
213	4	332	5
221	1	333	2
222	3		

As in the above, it has been confirmed that, if three
5 blocks of one gene are shuffled according to the method of the
present invention, a nucleic acid pool is obtained that covers
all combinations of clones each containing the same or
different three of these blocks.

10 Example 4: Screening of Genetic Products Obtained from Nucleic Acid Pool

The DNAs as produced in Example 3 were mixed. Using the
resulting DNA mixture, cells of *Bacillus subtilis* UOT0999 were
transformed. Tetracycline-resistant transformants were selec-
15 ted. 300 transformants were replicated on a skim milk-con-
taining medium plate, on which were found clear zones around
the colonies of 12 transformants. Accordingly, it is under-

stood that the enzyme which the shuffled gene codes for can be selected depending on its activity. The base sequences of these 12 clones that formed the clear zones were analyzed, from which it was found that these were sequenced in the same order of blocks as in the wild-type enzyme.

Example 5: Detection of Genetic Products

From 10 clones (one clone forms halo, while 9 clones do not) as selected from the transformants that had obtained in Example 3, and also from the host, *Bacillus subtilis* UOT0999, full-length RNAs were prepared. These were processed with a ribonuclease-free deoxyribonuclease, in order to remove the influence of the plasmids on the hybridization to be effected later on. Next, using oligo 1r as the probe, these were subjected to Northern hybridization. As a result, all lanes corresponding to the RNA of the transformants gave detectable bands, but no band was detected on the lanes corresponding to the RNA of the host.

Advantages of the Invention

According to the present invention, provided is a double-stranded DNA molecule with any desired cohesive end or ends. Using this, it is possible to obtain various DNAs with various base sequences which are substantially apart from the naturally-existing base sequence spaces, and also a DNA pool of a mixture of such DNAs, through simple processes. Therefore, it is possible to obtain excellent genetic products, such as proteins and enzymes, which could not be obtained in

conventional methods and which were not examined by organisms in the past. In addition, according to the method of the present invention for producing a nucleic acid pool, it is possible to obtain a mixture of nucleic acids while optionally
5 shuffling the constitutive blocks at random in the intermediate parts but fixing the terminal sequences to be predetermined, desired ones, and it is also possible to shuffle the constitutive blocks without changing the amino acid sequence which each block codes for. Therefore, as compared with a me-
10 thod of producing a completely-randomized nucleic acid pool, there is a high possibility that useful genetic products can be produced according to the method of the present invention.

5 Type of Sequence: Nucleic Acid

 Number of Strands: Double-stranded

 Topology: Linear

 Kind of Sequence: Genomic DNA

 Source: *Bacillus* NKS-21 (FERM BP-9

	ATG	AAT	CTT	CAA	AAA	ATA	GCC	TCA	GCG	TTG	AAG	GTT	AAG	CAA	TCG	GCA	48
	Met	Asn	Leu	Gln	Lys	Ile	Ala	Ser	Ala	Leu	Lys	Val	Lys	Gln	Ser	Ala	
20			-100					-95					-90				
	TTG	GTC	AGC	AGT	TTA	ACT	ATT	TTG	TTT	CTA	ATC	ATG	CTA	GTA	GGT	ACG	96
	Leu	Val	Ser	Ser	Leu	Thr	Ile	Leu	Phe	Leu	Ile	Met	Leu	Val	Gly	Thr	
		-85					-80					-75					
	ACT	AGT	GCA	AAT	GGT	GCG	AAG	CAA	GAG	TAC	TTA	ATT	GGT	TTC	AAC	TCA	144
25	Thr	Ser	Ala	Asn	Gly	Ala	Lys	Gln	Glu	Tyr	Leu	Ile	Gly	Phe	Asn	Ser	
	-70					-65					-60					-55	
	GAC	AAG	GCA	AAA	GGA	CTT	ATC	CAA	AAT	GCA	GGT	GGA	GAA	ATT	CAT	CAT	192
	Asp	Lys	Ala	Lys	Gly	Leu	Ile	Gln	Asn	Ala	Gly	Gly	Glu	Ile	His	His	
				-50						-45				-40			
30	GAA	TAT	ACA	GAG	TTT	CCA	GTT	ATC	TAT	GCA	GAG	CTT	CCA	GAA	GCA	GCG	240
	Glu	Tyr	Thr	Glu	Phe	Pro	Val	Ile	Tyr	Ala	Glu	Leu	Pro	Glu	Ala	Ala	
			-35					-30					-25				
	GTA	AGT	GGA	TTG	AAA	AAT	AAT	CCT	CAT	ATT	GAT	TTT	ATT	GAG	GAA	AAC	288
	Val	Ser	Gly	Leu	Lys	Asn	Asn	Pro	His	Ile	Asp	Phe	Ile	Glu	Glu	Asn	
35			-20					-15					-10				
	GAA	GAA	GTT	GAA	ATT	GCA	CAG	ACT	GTT	CCT	TGG	GGA	ATC	CCT	TAT	ATT	336
	Glu	Glu	Val	Glu	Ile	Ala	Gln	Thr	Val	Pro	Trp	Gly	Ile	Pro	Tyr	Ile	
		-5					1				5					10	

	TAC	TCG	GAT	GTT	GTT	CAT	CGT	CAA	GGT	TAC	TTT	GGG	AAC	GGA	GTA	AAA	384
	Tyr	Ser	Asp	Val	Val	His	Arg	Gln	Gly	Tyr	Phe	Gly	Asn	Gly	Val	Lys	
				15					20				25				
	GTA	GCA	GTA	CTT	GAT	ACA	GGA	GTG	GCT	CCT	CAT	CCT	GAT	TTA	CAT	ATT	432
5	Val	Ala	Val	Leu	Asp	Thr	Gly	Val	Ala	Pro	His	Pro	Asp	Leu	His	Ile	
				30				35					40				
	AGA	GGA	GGA	GTA	AGC	TTT	ATC	TCT	ACA	GAA	AAC	ACT	TAT	GTG	GAT	TAT	480
	Arg	Gly	Gly	Val	Ser	Phe	Ile	Ser	Thr	Glu	Asn	Thr	Tyr	Val	Asp	Tyr	
				45				50					55				
10	AAT	GGT	CAC	GGT	ACT	CAC	GTA	GCT	GGT	ACT	GTA	GCT	GCC	CTA	AAC	AAT	528
	Asn	Gly	His	Gly	Thr	His	Val	Ala	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	
				60				65					70				
	TCA	TAT	GGC	GTA	TTG	GGA	GTG	GCT	CCT	GGA	GCT	GAA	CTA	TAT	GCT	GTT	576
	Ser	Tyr	Gly	Val	Leu	Gly	Val	Ala	Pro	Gly	Ala	Glu	Leu	Tyr	Ala	Val	
15		75				80				85						90	
	AAA	GTT	CTT	GAT	CGT	AAC	GGA	AGC	GGT	TCG	CAT	GCA	TCC	ATT	GCT	CAA	624
	Lys	Val	Leu	Asp	Arg	Asn	Gly	Ser	Gly	Ser	His	Ala	Ser	Ile	Ala	Gln	
					95				100						105		
	GGA	ATT	GAA	TGG	GCG	ATG	AAT	AAT	GGG	ATG	GAT	ATT	GCC	AAC	ATG	AGT	672
20	Gly	Ile	Glu	Trp	Ala	Met	Asn	Asn	Gly	Met	Asp	Ile	Ala	Asn	Met	Ser	
				110					115					120			
	TTA	GGA	AGT	CCT	TCT	GGG	TCT	ACA	ACC	CTG	CAA	TTA	GCA	GCA	GAC	CGC	720
	Leu	Gly	Ser	Pro	Ser	Gly	Ser	Thr	Thr	Leu	Gln	Leu	Ala	Ala	Asp	Arg	
				125				130					135				
25	GCT	AGG	AAT	GCA	GGT	GTC	TTA	TTA	ATT	GGG	GCG	GCT	GGA	AAC	TCA	GGA	768
	Ala	Arg	Asn	Ala	Gly	Val	Leu	Leu	Ile	Gly	Ala	Ala	Gly	Asn	Ser	Gly	
				140				145					150				
	CAA	CAA	GGC	GGC	TCG	AAT	AAC	ATG	GGC	TAC	CCA	GCG	CGC	TAT	GCA	TCT	816
	Gln	Gln	Gly	Gly	Ser	Asn	Asn	Met	Gly	Tyr	Pro	Ala	Arg	Tyr	Ala	Ser	
30		155				160					165					170	
	GTC	ATG	GCT	GTT	GGA	GCG	GTG	GAC	CAA	AAT	GGA	AAT	AGA	GCG	AAC	TTT	864
	Val	Met	Ala	Val	Gly	Ala	Val	Asp	Gln	Asn	Gly	Asn	Arg	Ala	Asn	Phe	
					175					180					185		
	TCA	AGC	TAT	GGA	TCA	GAA	CTT	GAG	ATT	ATG	GCG	CCT	GGT	GTC	AAT	ATT	912
35	Ser	Ser	Tyr	Gly	Ser	Glu	Leu	Glu	Ile	Met	Ala	Pro	Gly	Val	Asn	Ile	
				190					195					200			
	AAC	AGT	ACG	TAT	TTA	AAT	AAC	GGA	TAT	CGC	AGT	TTA	AAT	GGT	ACG	TCA	960
	Asn	Ser	Thr	Tyr	Leu	Asn	Asn	Gly	Tyr	Arg	Ser	Leu	Asn	Gly	Thr	Ser	
				205				210					215				
40	ATG	GCA	TCT	CCA	CAT	GTT	GCT	GGG	GTA	GCT	GCA	TTA	GTT	AAA	CAA	AAA	1008
	Met	Ala	Ser	Pro	His	Val	Ala	Gly	Val	Ala	Ala	Leu	Val	Lys	Gln	Lys	
				220				225					230				
	CAC	CCT	CAC	TTA	ACG	GCG	GCA	CAA	ATT	CGT	AAT	CGT	ATG	AAT	CAA	ACA	1056
	His	Pro	His	Leu	Thr	Ala	Ala	Gln	Ile	Arg	Asn	Arg	Met	Asn	Gln	Thr	
45		235				240					245					250	
	GCA	ATT	CCG	CTT	GGT	AAC	AGC	ACG	TAT	TAT	GGA	AAT	GGC	TTA	GTG	GAT	1104
	Ala	Ile	Pro	Leu	Gly	Asn	Ser	Thr	Tyr	Tyr	Gly	Asn	Gly	Leu	Val	Asp	
					255					260				265			
	GCT	GAG	TAT	GCG	GCT	CAA											1122
50	Ala	Glu	Tyr	Ala	Ala	Gln											
				270		272											

Sequence Number: 2

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

5 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GATTTTAGAA TTCGCAGCGG

10 Sequence Number: 3

Length of Sequence: 25

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

15 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CCGGATTCCT TAAAGCCCTG AATAA

Sequence Number: 4

20 Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

25 Sequence:

ACAGTCTGTG CAATTTC

Sequence Number: 5

Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

5 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GAAATTGCAC AGACTGT

Sequence Number: 6

10 Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

15 Sequence:

CCTTGGGGAA TCCCTTATAT

Sequence Number: 7

Length of Sequence: 17

20 Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

25 CCCAATACGC CATATGA

Sequence Number: 8

Length of Sequence: 17

Type of Sequence: Nucleic Acid

5 Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

TCATATGGCG TATTGGG

10

Sequence Number: 9

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

15 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GTGGCTCCTG GAGCTGAACT

20 Sequence Number: 10

Length of Sequence: 16

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

25 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

TCTGATCCAT AGCTTG

Sequence Number: 11

Length of Sequence: 16

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

5 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CAAGCTATGG ATCAGA

10 Sequence Number: 12

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

15 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CTTGAGATTA TGGCGCCTGG

Sequence Number: 13

20 Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

25 Sequence:

TGAGCCGCAT ACTCAGC

Sequence Number: 14

Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

5 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GCTGAGTATG CGGCTCA

Sequence Number: 15

10 Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

15 Sequence:


TAATCCCTAA GGATGTACTG

Brief Description of the Drawing

Fig. 1 is a graphical view showing one embodiment of the method of
20 the present invention for shuffling a DNA.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 24 , lines 12-15 to page lines	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry	
Address of depositary institution (including postal code and country) 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan	
Date of deposit 7 May 1985	Accession Number FERM BP-93-1
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only
<input checked="" type="checkbox"/> This sheet was received with the international application
Authorized officer  Anne-Grethe Warrer-Madsen Head Clerk

For International Bureau use only
<input type="checkbox"/> This sheet was received by the International Bureau on: _____
Authorized officer

CLAIMS

1. A DNA with a cohesive end comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, and (b) a single-stranded DNA having a base sequence that
5 exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, wherein the single-stranded DNA is linked to either one end of the double-stranded DNA to form a cohesive end.

10 2. A DNA with cohesive ends comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, (b) a first, single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence
15 which said gene does not have, and (c) a second, single-stranded DNA having a base sequence that exists on said gene at the site adjoining the part corresponding to said double-stranded DNA, wherein the second, single-stranded DNA is linked to said double-stranded DNA at one end corresponding to said
20 adjoining site, while the first, single-stranded DNA is linked thereto at the other end of the complementary strand opposite to said end, thereby forming cohesive ends.

3. The DNA with a cohesive end or cohesive ends as claimed in claim 1 or 2, wherein the single-stranded DNA has a
25 length of 2 bases or more.

4. The DNA with a cohesive end or cohesive ends as claimed in any one of claims 1 to 3, wherein the cohesive end/ends is/are positioned at the 3'-terminal/terminals.

5. A method for producing a DNA with a cohesive end or cohesive ends, wherein a part of a DNA, as a template, and an oligonucleotide containing at least one ribonucleotide, as a primer, are subjected to DNA polymerase reaction to prepare a double-stranded DNA, then the ribonucleotide(s) is/are removed through enzymatic reaction or chemical reaction, and the nucleotide(s) remaining at the 5'-terminal(s) of the site(s) at which said ribonucleotide(s) existed are removed.

6. A method for producing the DNA with a cohesive end as set forth in claim 1, comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and the linked oligonucleotide as obtained in the previous step a), as a primer;

c) a step of removing the ribonucleotide from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotide remaining at the 5'-terminal of the site at which said ribonucleotide existed.

7. A method for producing the DNA with cohesive ends as set forth in claim 2, comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and (i) the linked oligonucleotide as obtained in the previous step a) and (ii) an oligonucleotide which is a complementary strand of an oligonucleotide existing on the gene at the site separated from said oligonucleotide-corresponding part by at least 3 bases or more toward the 3'-terminal and which contains at least one ribonucleotide, as primers;

c) a step of removing the ribonucleotides from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotides remaining at the 5'-terminals of the sites at which said ribonucleotides existed.

8. A method for shuffling a DNA, comprising dividing a DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, followed by ligating them together into a

sequence that is different from the sequence of the original, non-divided DNA.

9. A method for shuffling a DNA, comprising applying the method as set forth in any one of claims 5 to 7 to various
5 sites of a DNA, thereby dividing the DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, at least one block of which shall have a cohesive end that is complementary to the cohesive end of another block not having been directly adjacent to said one block on the original DNA,
10 followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

10. The shuffling method as claimed in claim 8 or 9, wherein the DNA is divided into 3 or more blocks.

11. The shuffling method as claimed in any one of claims
15 8 to 10, wherein the blocks are ligated together using a DNA ligase.

12. A DNA as shuffled according to the method as set forth in any one of claims 8 to 11.

13. The DNA as claimed in claim 12, wherein a gene coding
20 for an enzymatic function or a control gene for the gene is shuffled.

14. The DNA as claimed in claim 13, wherein the gene is a gene that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases,
25 oxygenases and reductases.

15. The DNA as claimed in claim 13 or 14, wherein the gene is one derived from prokaryotes.

16. The DNA as claimed in claim 15, wherein the gene is one derived from bacillus bacteria.

17. The DNA as claimed in claim 16, wherein the gene is a protease API21 gene.

5 18. A DNA pool containing plural kinds of DNAs having different structures that are obtained according to the shuffling method as set forth in any one of claims 8 to 11.

19. The DNA pool as claimed in claim 18, which contains 10 or more kinds of DNAs.

10 20. A method for producing a DNA pool, comprising applying the method as set forth in any one of claims 5 to 7 to various sites of a template DNA to thereby prepare a mixture of DNA blocks each having a cohesive end or cohesive ends that satisfies the following conditions, followed by ligating these
15 into any desired sequences:

Condition 1: Each block has a double-stranded site having the same sequence as that of a part of the template DNA.

Condition 2: At least two of the blocks that constitute the block mixture further have, in addition to said double-
20 stranded site, a single-stranded site (cohesive end) that is complementary to the cohesive end of blocks that are not directly adjacent to said blocks on the template DNA.

Condition 3: The block mixture contains at least two different blocks which are the same in the double-stranded site
25 but are different only in the single-stranded site and which satisfy the condition 2.

21. The method for producing a DNA pool as claimed in claim 20, wherein the template DNA is a gene that codes for an enzymatic function or a control gene DNA for the gene.

22. The method for producing a DNA pool as claimed in
5 claim 21, wherein the template DNA is a gene DNA that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

23. The method for producing a DNA pool as claimed in claim 22, wherein the template DNA is one derived from
10 prokaryotes.

24. The method for producing a DNA pool as claimed in claim 23, wherein the template DNA is one derived from bacillus bacteria.

25. The method for producing a DNA pool as claimed in
15 claim 24, wherein the template DNA is a protease API21 gene.

26. The method for producing a DNA pool as claimed in any one of claims 20 to 25, wherein the DNA blocks are ligated together using a DNA ligase.

27. A genetic product to be obtained by expressing the
20 genetic information on DNA molecules that exist in the DNA pool as set forth in any one of claims 18 to 26.

1/1

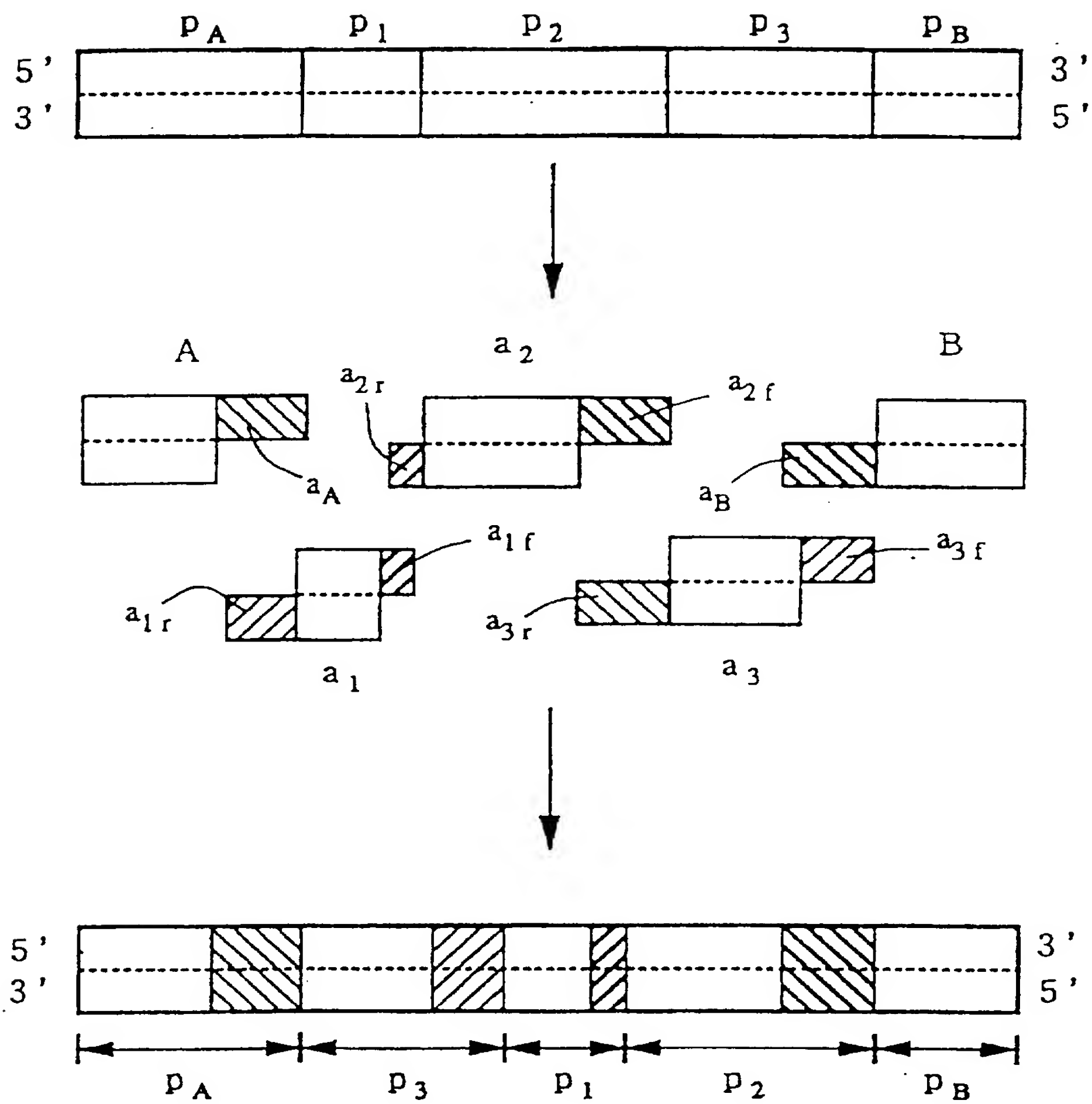


Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/10, C12Q 1/68 // C12N 9/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, DBA, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemistry Letters, Volume 2, 1995, Koichi Nishigaki et al, "Restriction-Enzyme-Nondependent Recombination and Rearrangement of DNA (RRR)" page 131 --	1-27
X	WO 9107506 A1 (UNITED STATES OF AMERICA), 30 May 1991 (30.05.91), fig. 7 and the whole document --	1-4
X	WO 9517413 A1 (EVOTEC BIOSYSTEMS GMBH), 29 June 1995 (29.06.95), the whole document, see especially page 8, line 3-7, page 9, line 15-16, page 16, line 7-11 and claims --	8-19,27

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 "&" document member of the same patent family

Date of the actual completion of the international search

21 November 1997

Date of mailing of the international search report

01-12-1997

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 9522625 A1 (AFFYMAX TECHNOLOGIES N.V.), 24 August 1995 (24.08.95), the whole document, see especially page 44, line 34 - page 45, line 15</p> <p>-- -----</p>	8-19,27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

According to PCT rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

Such a unifying link would be a DNA sequence with a cohesive end comprising a double stranded DNA sequence from a gene linked to at least one single stranded DNA not adjoining the double stranded DNA sequence in the gene. However such a DNA sequences are known in the art see e.g. WO9107506 or Nishigaki et.al in the search report. No other unifying special technical feature have been found.

The application is considered to comprise of the following inventions:

Invention 1, claims 1-4: DNA sequence with a cohesive end comprising a double stranded DNA sequence from a gene linked to at least one single stranded DNA not adjoining the double stranded DNA sequence in the gene.

Invention 2, claims 5-7 and 20-26, and related parts of claim 27: A method for producing DNA sequence with cohesive ends using ribonucleotides as a primer to create a double stranded DNA with polymerase reaction, whereafter the ribonucleotides are removed to create a cohesive end and a method for producing a DNA pool by applying the method.

Invention 3, claim 8-19 and related parts of claim 27: A method for shuffling a DNA and a DNA pool containing DNAs obtained by the method.

In spite of the non-unity all claims have been searched.

INTERNATIONAL SEARCH REPORT

01/10/97

International application No.

PCT/DK 97/00317

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9107506	A1	30/05/91	AU	6886991 A	13/06/91
WO	9517413	A1	29/06/95	DE	4343591 A	22/06/95
WO	9522625	A1	24/08/95	AU	2971495 A	04/09/95
				CA	2182393 A	24/08/95
				CN	1145641 A	19/03/97
				EP	0752008 A	08/01/97
				US	5605793 A	25/02/97